this reaction unless one of the groups is alkyl.

A number of the aromatic nitro amines have been reduced to the corresponding polyamines.

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Physical, Chemical and Immunological Properties of Phosphorylated Crystalline Horse Serum Albumin[1]

BY MANFRED MAYER AND MICHAEL HEIDELBERGER

The decisive importance of phosphorylation in synthesis and the transfer of energy in biological processes suggested the introduction of phosphoryl groups into proteins under the mildest possible conditions and a study of the physical, chemical, and immunological changes produced by their introduction. Initial experiments were carried out with crystalline egg albumin,[2] but since it appeared that denaturation accompanied the phosphorylation of this protein, the present study with crystalline horse serum albumin (SA) was undertaken in the hope of avoiding this complication.

Several preparations of phosphorylated serum albumin (PSA) of different P content were made by Rimington's method,[3] as well as by a milder modification in which sodium borate or, preferably, potassium borate was substituted for sodium hydroxide.

Preparation and Chemical and Physical Properties of Phosphorylated Serum Albumin

1. **Preparation of SA.**—Horse serum albumin, crystallized by Adair and Robinson's method[4] and recrystallized five times, was dialyzed against 0.9% sodium chloride solution until free from ammonium sulfate, and then against distilled water until free from chloride ion. After sterilization by filtration through a Chamberland L2 candle, the solution was stored in the refrigerator without preservative. No deterioration was apparent during two years. In contrast to native horse serum, from which the albumin is readily crystallized only when fresh, one purified lot was easily recrystallized after six months of storage. A typical preparation of SA contained 0.05% carbohydrate estimated as galactose by the orcinol method.[5] One-hundred and forty mg. contained 0.18% ash as sodium sulfate, but no phosphorus by the Pregl–Lieb method. The anhydrous protein was obtained by pouring a portion of the aqueous serum albumin solution into 10 vols. of redistilled alcohol and washing the resulting precipitate with alcohol and acetone. After filtration, the precipitate was dried to constant weight at room temperature *in vacuo* over phosphorus pentoxide. Nitrogen was estimated by a modification of the micro-Kjeldahl procedure. The nitrogen content of 15.82% obtained in this way was in good accord with the value, 15.86%, found for another lot which was dried *in vacuo* over phosphorus pentoxide at room temperature directly from the aqueous solution.

2. **Phosphorylation of SA and Isolation of PSA.**—In a typical phosphorylation (PSA3), 1.5 g. of SA dissolved in 55 ml. of water and 55 ml. of 6% $Na_2HPO_4 \cdot 12H_2O$ solution were placed in a 500-ml., three-necked flask immersed in an ice–salt–water bath kept at 0 to −2°. Three grams of freshly distilled phosphorus oxychloride dissolved in 25 ml. of carbon tetrachloride were added dropwise over a period of six hours to the mechanically stirred mixture. Simultaneously, 3 *N* sodium hydroxide solution was added drop by drop to keep the reaction mixture slightly alkaline to phenolphthalein, indicating a *p*H of 8.5 to 9. Since it was feared that the use of strong alkali had caused excessive denaturation in such preparations, solid sodium or potassium borate was substituted in later runs. After addition of the reagents the reaction mixture was placed in the refrigerator until the following day when it was centrifuged in the cold[6] to separate the semi-solid carbon tetrachloride emulsion from the aqueous phase containing the PSA. Lots prepared with sodium hydroxide could be precipitated by addition of *N* hydrochloric acid or preferably acetic acid to about *p*H 3 to 4. After dispersing the centrifuged precipitate in cold water or 0.9% sodium chloride solution the product was dissolved by addition of *N* sodium hydroxide to *p*H 5 to 6 and the isoelectric precipitation was usually repeated. Precipitation was omitted in the case of the less heavily phosphorylated and therefore more soluble lots in the preparation of which potassium or sodium borate had been used. The free phosphate of all lots was reduced to a minimum, usually a few tenths of a μg of phosphorus per ml., by dialysis in the cold against isotonic saline. Traces of phosphate continued to appear in the dialyzate indefinitely, indicating slow spontaneous splitting from the phosphoprotein. The resultant decline in phosphorus content with age could be minimized by storage at *p*H 8 to 9 in the refrigerator or, better, in solid carbon dioxide. The products were analyzed for nitrogen by the micro-Kjeldahl method and for phosphorus after Pregl–Lieb, and the N:P ratios were calculated.

The mode of preparation and purification as well as the properties of all lots of PSA and their fractionation products are described in Table I. Unless otherwise stated, separation and purification of the phosphoprotein was carried out one day after phosphorylation.

3. **Spontaneous Loss of Phosphorus.**—After dialysis to remove inorganic phosphate and analysis for nitrogen and phosphorus, portions of various PSA lots were stored for varying periods and at different temperatures and were then dialyzed and analyzed for phosphorus. In some cases the phosphate which had been split off was determined colorimetrically[7] in the dialyzate.

At 37°, in the presence of toluene, dephosphorylation took place rapidly, especially in slightly acid media. In thirteen days, at *p*H 7.4, PSA4 lost 35 of its 48 phosphoryl groups (*cf.* 4E) (calculated from nitrogen and phosphorus contents on the basis of a molecule of original serum al-

(1) The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital and was submitted by Manfred Mayer in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(2) M. Heidelberger, B. Davis and H. P. Treffers, THIS JOURNAL, **63**, 498 (1941).

(3) C. Rimington, *Biochem. J.*, **21**, 272 (1927).

(4) G. S. Adair and M. E. Robinson, *ibid.*, **24**, 993 (1930).

(5) M. Heidelberger and F. E. Kendall, *J. Immunol.*, **30**, 267 (1936).

(6) In an International Equipment Company refrigerated centrifuge.

(7) A. Bodansky, *J. Biol. Chem.*, **99**, 197 (1932–1933).

TABLE I

PREPARATION AND PROPERTIES OF PSA

1	2	3	4	5	6	7	8	9	10	11	12
Prepn.	$POCl_3$: SA weight ratio	Neutralizing agent used	Isolation procedure	N:P weight ratio of product	P atoms per mol., wt. 70,000	Relative fluidity at 1% protein concn.	Cross reaction with 1.0 ml. anti-SA rabbit serum. N precipitated by 0.05 mg. of antigen N, mg.	Cross reaction with 1.0 ml. anti-SA rabbit serum. N precipitated by 0.10 mg. of antigen N, mg.	Ratio of PSA–Folin color to SA–Folin color	Ratio of Van Slyke amino N to total N	Number of blocked amino groups per mol. wt. 70,000
1	1:2	*N* NaOH	5 iep. pptns., dial.[a]	10.5	34						
2	1:2	*N* NaOH	5 iep. pptns.	10.2	35						
2′	1:2	*N* NaOH	Partially dephosphd. in icebox	23.1	15		0.46	0.66			
3	2:1	3 *N* NaOH	$(NH_4)_2SO_4$, iep. pptn.[b]	11.2	32						
4A[c]	1:2	*N* NaOH	5 iep. pptns., dial.	7.3	48						
4B[d]	1:2	*N* NaOH	5 iep. pptns., dial.	9.0	40	0.896	.26	.46			
$4C_1$[e]	1:2	*N* NaOH	[e]	13.5	26	.922	.40	.52	0.65		
$4C_2$[e]	1:2	*N* NaOH	[e]	13.5	26		.46	.60	.70		
4D[f]	1:2	*N* NaOH	2 iep. pptns., dial.	9.1	39	.898	.36	.44	.66		
$4E_1$[g]	1:2	*N* NaOH	[g]	25.0	14	.918	.38	.53	.63		
$4E_2$[g]	1:2	*N* NaOH	[g]	27.6	13		.45	.58	.71		
$4E_3$[g]	1:2	*N* NaOH	[g]	32.4	11	.944	.44	.61	.70		
5B[h]	2:1	2 *N* NaOH	2 iep. pptns., dial.	7.2	49	.860	.20	.22	.25		
$5E_1$[h]	2:1	2 *N* NaOH	[h]	15.3	23	.886	.27	.36	.24		
$5E_2$[h]	2:1	2 *N* NaOH	[h]	15.7	23		.28	.34	.32		
$5E_3$[h]	2:1	2 *N* NaOH	[h]	16.2	22	.952	.26	.30			
6	1:2	$Na_2B_4O_7$	Dialysis	21.6	17						
7	1:2	$Na_2B_4O_7$	Dialysis	20.6	17		.51	.73			
8	1:2	$K_2B_4O_7$	Dialysis	14.6	24			.69	.92		
10S[i]	1:1	$K_2B_4O_7$	[i]	14.7	24	.942	.49	.69	.90		
10P	1:1	$K_2B_4O_7$		13.3	27	.920	.47	.65	.89		
11S[i]	1:2	$K_2B_4O_7$	[i]	18.7	19				.85		
11P	1:2	K_2CO_3		16.5	22						
13	2:1	2 *N* NaOH	Dialysis	5.9	61		.18	.14	.36	0.043	27
13A[j]	2:1	2 *N* NaOH	Dialysis	24.7	14		.35	.47	.44	.059	15
14	1:1	$K_2B_4O_7$	Dialysis	11.2	32	[k]					
SA						.962	.62	.86	1.00	.078[28]	

[a] Five successive isoelectric precipitations, followed by dialysis of redissolved ppt. [b] Since the isoelectric point in the crude state was near *p*H 2 it was feared that precipitation with acid of this strength might denature the protein; 1 vol. of saturated $(NH_4)_2SO_4$ solution was added and the PSA separated when the *p*H was brought to 4.2. After solution, PSA3 was precipitated 4 times between *p*H 3 and 4. [c] Purified immediately after phosphorylation. [d] Separated from the reaction mixture after one month at *p*H 9.10 in the icebox. [e] Isolated from the reaction mixture after two months at *p*H 9.10 in the icebox. After dialysis for removal of phosphate the product was acidified to *p*H 5.30 and again stored in the icebox for nearly two months. It was then separated into insoluble and soluble fractions (C_1 and C_2) at the iep. and dialyzed. [f] Separated after four months at *p*H 9.10 in the icebox. Properties almost identical with those of 4B isolated three months earlier. [g] Isolated after four and one-half months; kept thirteen days at *p*H 7.4 and 37°, and then separated into fractions of low, intermediate and high solubility at the iep. (E_1, E_2, E_3). [h] Analogous to corresponding PSA4 fractions. [i] Lots 10 and 11 were separated into isoelectrically soluble (S) and insoluble (P) fractions. [j] From a solution of PSA13 let stand thirteen days at 37° and *p*H 5.5. [k] The relative fluidities at 1% protein concentration were 0.902 and 0.922 in sodium chloride soln., $\mu = 0.05$ and $\mu = 0.2$, respectively.

bumin of molecular weight 70,000). Portions of PSA3 kept for seven days at 37° and *p*H 8.3, 6.7 and 4.9 lost 38, 47 and 53% of their bound phosphorus, respectively. PSA13, which was also made with sodium hydroxide for neutralization, when kept in *p*H 5.3 acetate–buffer at 37°, lost almost two thirds of the bound phosphoryl groups in three days.

Even during storage in the refrigerator, PSA4 lost 22 of its 48 phosphorus atoms (*cf.* 4C) (per molecule of original serum albumin) in two months at *p*H 5.3, while another aliquot kept at *p*H 8 to 9 lost only 8 phosphorus atoms in one month (*cf.* 4B) and remained stable during another three months under the same conditions (*cf.* 4D). By storing the slightly alkaline solution of PSA13, also a sodium hydroxide preparation, at about −70°, in frozen carbon dioxide, spontaneous dephosphorylation was arrested completely for three and one-half months at a high level of 60 phosphoryl groups.

The partly dephosphorylated derivatives from borate preparations retained much less phosphorus than their analogs prepared in the presence of sodium hydroxide. After 320 days in the icebox at *p*H 7, PSA8 held merely 7 atoms of phosphorus. Subsequent treatment with 1% sodium hydroxide at 37° for twenty-four hours[8] failed to split off this remainder. The aliquot of PSA3 which had been partly dephosphorylated at *p*H 8.3 likewise resisted treatment with 1% sodium hydroxide at 37° for twenty-four hours.

4. Homogeneity of PSA.—Since SA is soluble throughout the entire *p*H range, its absence in acid-precipitated PSA was assumed, but in the case of borate–PSA, in which appreciable solubility at all acidities precluded extensive purification by isoelectric precipitation, characterization

(8) R. H. A. Plimmer and W. M. Bayliss, *J. Physiol.*, **33**, 439 (1905–1906).

in the Tiselius electrophoresis apparatus was used as a control.[9] PSA10A, PSA10B and PSA14 (see Table I) were studied in this way and found to be essentially free from SA.

Evidence of homogeneity in an isoelectric-precipitable lot was secured by fractional acid precipitation of PSA2, which had been purified by five successive isoelectric precipitations followed by two days of dialysis to remove inorganic phosphate. Three hundred mg. of protein was successively adjusted to *p*H 4.63, 4.40 and 4.05. The resulting precipitates were separated at each step and analyzed for nitrogen and phosphorus. The results, tabulated below, indicate homogeneity with respect to the extent of phosphorylation but not necessarily in regard to the distribution of phosphoryl radicals on the reactive groups of SA, presumably aliphatic and aromatic OH, α- and ϵ-NH_2, imidazole and guanidino-groups.

TABLE II

*p*H of pptn.	Mg. protein pptd.	N:P ratio of ppt.
4.63	63	15.5
4.40	160	15.5
4.05	37	15.6

Out of 300 mg. of protein only 260 were precipitated.

N:P ratios of soluble and insoluble fractions could, however, be compared directly in other cases. A portion of PSA4 was precipitated three times with acid, dialyzed free from inorganic phosphate, adjusted to *p*H 5.3 and stored in the refrigerator for 55 days to permit splitting of labile phosphate. This was removed by dialysis. Isoelectric precipitation at *p*H 3.86 of the resulting "stabilized" product, of lower P content than the original PSA4, yielded soluble and insoluble fractions, both of which showed N:P = 13.4. PSA3 showed similar behavior.

Fractionation of PSA10 and PSA11, both prepared without the use of strong alkali, revealed small N:P differences:

TABLE III

N:P RATIOS OF INSOLUBLE AND SOLUBLE PSA FRACTIONS

	PSA10		PSA11
At the isoelectric point	Per cent. of total protein	N:P	N:P
Fraction insoluble	77	13.3	16.5
Fraction soluble	23	14.7	18.7

A more elaborate fractionation was carried out with PSA4E, which was dialyzed in the cold until free from phosphate ion, and adjusted with *N* hydrochloric acid to *p*H 4.3. The bulky precipitate was centrifuged off, redissolved with the aid of *N* sodium hydroxide and reprecipitated twice more with hydrochloric acid. The insoluble fraction, E_1, comprised 48% of the total. The supernatants from the three isoelectric precipitations were combined and the protein which they contained was completely precipitated by half-saturation with sodium sulfate at *p*H 4 to 5 and dissolved in a volume of 30 ml. (approximately $^1/_3$ the volume of the combined supernatants), dialyzed to equilibrium against 0.9% sodium chloride solution, and brought to *p*H 4.3 with *N* hydrochloric acid. A small amount of precipitate (7% of the total protein), representing a fraction of intermediate solubility, was separated (E_2). The supernatant, E_3, contained 45% of the total protein. While incubation for 13 days had split off about one-half of the bound phosphorus, the N:P ratios of the three fractions (see Table I) differed only slightly in spite of widely divergent solubilities, leading to the conclusion that PSA4 was largely homogeneous in regard to phosphorus content, but not in regard to solubility. A similar experiment was carried out with the more heavily phosphorylated PSA5, yielding three fractions, E_1, E_2 and E_3, of low, medium and high solubility, but with almost identical N:P ratios.

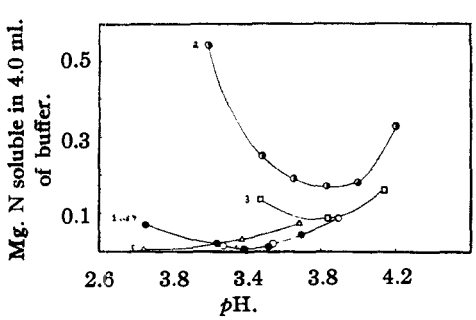


Fig. 1.—Solubility of PSA in 0.1 *M* citrate-HCl buffer: curve 1 (solid circles), prepn. 4B, 0.74 mg. PSA N used; curve 2, prepn. 4C. 0.90 mg. N; curve 3 (open squares), prepn. 4C′, 0.69 mg. N; curve 4 (open circles), prepn. 4D, 0.48 mg. N; curve 5 (open triangles), prepn. 5B, 0.57 mg. N.

(9) Electrophoresis and sedimentation runs were carried out by Dr. Dan H. Moore.

5. *p*H-Dependence of Solubility of PSA.—Quantitative solubility determinations at different *p*H values throughout the isoelectric range were carried out in several instances. 1.0-ml. portions of a solution of PSA containing 0.5 to 1 mg. of nitrogen per ml. were mixed in the cold with 3.0 ml. of 0.1 *M* citrate–hydrochloric acid buffer at *p*H values in the isoelectric range (*p*H 2.5 to 4.5). After eight hours in the cold the tubes were spun in a refrigerated centrifuge, the supernatants were decanted as completely as possible, and the precipitates were analyzed by the micro-Kjeldahl nitrogen method. The *p*H of each supernatant was measured with a glass electrode. A solubility curve (Fig. 1) was drawn from the data without regard to the slight error introduced by analyzing the wet precipitate instead of the supernatant.

A product such as PSA5B, which was made with sodium hydroxide and contained much phosphorus, showed a more acidic isoelectric point than the less heavily phosphorylated lots. Phosphorus loss, as shown in the partial dephosphorylation of PSA4B to PSA4C, was accompanied by a shift of the isoelectric point toward higher *p*H as well as by a rise in solubility (Fig. 1, curves 1 and 2).

6. Reactivity of PSA with the Folin–Ciocalteau Phenol Reagent.—The color intensity given by PSA with the Folin–Ciocalteau reagent[10] was always less than produced by SA (*cf.* Table I, column 10). Those preparations which had been made with the aid of sodium hydroxide (lots 4, 5, 13) gave less color with the Folin reagent than did the ones prepared with borate (lots 8, 10, 11). Lots 5 and 13, which were made with a large excess of phosphorus oxychloride ($POCl_3$:SA = 2:1), yielded even less color than did lot 4, which had been treated with only a moderate amount of phosphorus oxychloride ($POCl_3$:SA = 1:2). SA (1 mg. of nitrogen per ml.) in 1 *N*, 2 *N* and 3 *N* sodium hydroxide for three and three-fourths hours at 0–2° showed the opposite effect, giving 25% greater color intensity. This was also noted with egg albumin (Ea) denatured by sodium hydroxide. The decrease of color reactivity in the case of PSA may therefore be attributed to some phase of the reaction with phosphorus oxychloride. Following the lead of earlier workers,[11,12,13] the decrease of color was taken as evidence of tyrosine esterification, but quantitative evaluation of the number of tyrosine groups blocked was not attempted because other factors are known to be operative. Besides the increase due to denaturation, it has been reported that peptides of tyrosine give less color than does tyrosine.[14] Moreover, PSA13B, which

(10) O. Folin and V. Ciocalteau, *J. Biol. Chem.*, **73**, 627 (1927).

(11) R. M. Herriott, *J. Gen. Physiol.*, **19**, 283 (1935).

(12) W. F. Ross and H. N. Christensen, *J. Biol. Chem.*, **137**, 89 (1941).

(13) G. L. Miller and W. M. Stanley, *ibid.*, **141**, 905 (1941).

(14) G. L. Miller, *ibid.*, **146**, 345 (1942).

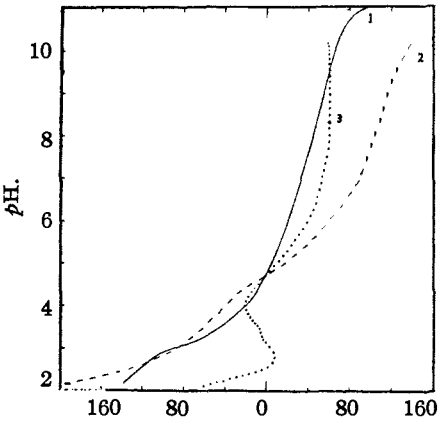
had been dephosphorylated to 6 atoms of phosphorus per molecule of SA still reacted with the Folin reagent as weakly as if 11 tyrosine residues were esterified. This might suggest the existence of phosphoryl bridges between two tyrosine residues. Dephosphorylation, in general, resulted in only moderate increases of Folin reactivity. While the insoluble fractions ($4C_1$, $4E_1$, $5E_1$) of dephosphorylated products showed no increase in color values the more soluble fractions ($4C_2$, $4E_2$, $4E_3$, $5E_2$) yielded somewhat more color after dephosphorylation. Apparently most of the phosphoryl groups split off were bound to groups other than tyrosine.

7. Phosphorylation of Amino Groups.—The volumetric Van Slyke method[15] for amino-nitrogen was employed to estimate phosphamide groups in PSA. Deaminations were run at 24–26° for fifteen minutes. When SA is treated with nitrous acid in this way a quantity of nitrogen corresponding to 62 ± 2 amino groups per molecule of SA is liberated. On analysis of the most heavily phosphorylated preparation, PSA13, which carried 61 phosphoryl groups, nitrogen accounting for only 34 ± 2 amino groups was liberated, indicating that some 28 amino groups did not react. Twenty-eight of the 61 phosphoryl groups are therefore either linked to amino groups or some or all of the 28 amino groups are prevented from reacting by neighboring phosphoryl groups. Actually, many more may have been bound in this way since it is probable that dephosphorylation took place during the analysis. Nitrous acid treatment of PSA13A, which had been stabilized at a phosphorus content of 14 atoms, liberated nitrogen equivalent to 47 amino groups. Therefore 15 amino groups were blocked, showing that all of the 14 phosphoryl groups of PSA13A were in N–P linkage or near NH_2 groups. One may, therefore, conclude that those phosphoryl groups which resist spontaneous dephosphorylation, as well as some of the labile ones, are either bound to amino groups or shield them from reaction with nitrous acid, while the rest of the labile ones may or may not be held in this type of linkage.

8. Titration of PSA with Acid and Alkali.—For each N–P linkage which is established, or each NH_2 otherwise blocked, the protein loses one basic radical and gains two acidic groups. This change, of course, finds expression in the course of the titration curve.[16]

About 180 mg. of PSA4B in 5 ml. was dialyzed against 0.15 *N* potassium chloride in the cold. The *p*H was then adjusted to 5.9 with 0.1 *N* hydrochloric acid and the solution kept in a partially exhausted desiccator in the presence of 0.15 *N* potassium hydroxide to remove carbon dioxide. The next day, 5 ml. portions of the carbon dioxide-free solution at *p*H 6.01, containing 47 mg. of protein, were titrated in N_2 with 0.02 ml. increments of 0.1 *N* hydrochloric acid and 0.1 *N* carbonate-free potassium hydroxide, using the differential technique with glass electrodes,[17] one of which was kept constant at *p*H 6.95 with buffer solution. Nitrogen and phosphorus analyses were carried out after the titration, as well as a Van Slyke carbon dioxide determination to ascertain absence of carbonate. In the acid range of the titration curve precipitation began near *p*H 4.4 and re-solution commenced as *p*H 3 was approached; possibly equilibrium was not attained in the precipitating range.

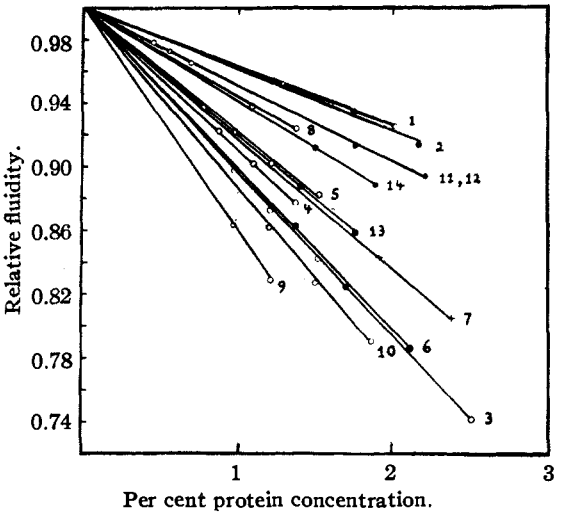
For comparison, the titration curve of SA[18] was superimposed on the PSA curve (Fig. 2), plotting the equivalents of H^+ combined with 1 g. of SA and with 1.04 g. of PSA4B (4.4% phosphoryl content). Subtraction of the values of the combined equivalents in the SA curve from those at the same *p*H values in the PSA curve, gives the comparison



Equivalents H^+ ($\times 10^5$) bound per gram of protein.

Fig. 2.—Titration curves of SA (solid line, curve 1) and PSA4B (dashes, curve 2) with acid and alkali. Curve 3 (dots) is the comparison curve obtained by subtraction of the values of the combined equivalents in the SA curve from those of the same *p*H values in the PSA curve. The isoelectric point of SA (*p*H 4.9) is used as the point of origin of the curves.

curve which shows approximately 60×10^{-5} equivalents of additional combining groups between *p*H 5 and 8, and another 60×10^{-5} additional equivalents between *p*H 2 and 2.5. The irregular part of the curve between *p*H 2.5 and 4.5 may be disregarded because PSA is insoluble in this range. Since the number of phosphoryl equivalents found by analysis is 56×10^{-5}, the titration data show the introduction of two base-combining groups per phosphorus



Per cent protein concentration.

Fig. 3.—Relative fluidities of SA and PSA solutions. In saline, unbuffered, μ = 0.15: line 1 (open circles), SA at *p*H 4.9; line 2 (solid circles), SA at *p*H 6.6; line 3 (open circles), PSA4B at *p*H 6.6; line 4, PSA4C′ at *p*H 7.6. At *p*H 6.6, in saline, unbuffered, μ = 0.15: line 5 (open circles), PSA4C′; line 6 (solid circles), PSA4D. At *p*H 6.6, in saline-phosphate buffer, μ = 0.17: line 7 (crosses) $PSA4E_1$; line 8 (open circles), $PSA4E_3$; line 9, PSA5B; line 10, $PSA5E_1$; line 11 (solid circles), $PSA5E_3$; line 12 (open circles), PSA7. At *p*H 8.0, borate-NaCl buffer, μ = 0.16: line 13 (solid circles) PSA10P; line 14 (solid circles) PSA10S.

(15) D. D. Van Slyke, *J. Biol. Chem.*, **9**, 185 (1911); **12**, 275 (1912); **16**, 121 (1913–1914).

(16) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 446.

(17) D. A. MacInnes, "The Principles of Electrochemistry," Reinhold Publ. Corp., New York, N. Y., 1939, p. 306.

(18) C. L. A. Schmidt, "The Chemistry of the Amino Acids and Proteins," C. C. Thomas, Springfield, Ill., Baltimore, Md., 1938, p. 726.

atom, indicating the exclusive formation of mono-esters of phosphoric acid.

9. Viscosity and Relative Fluidity of PSA.—Two and five-tenths ml. of a 2 to 4% solution of PSA at *p*H 6.6, unless otherwise stated in Fig. 3, were dialyzed against 0.9% sodium chloride solution in the cold and readjusted to *p*H 6.6, if necessary. In some cases the protein was dialyzed against saline buffered with phosphate or borate, as stated in the legend for Fig. 3. The viscosities of the solutions and of several dilutions, were measured at 25 ± 0.05° in an Ostwald viscometer of 2-ml. capacity. The densities of each dilution were determined by weighing 1.5 or 2.0 ml. immediately after measurement of the viscosity. Instead of relative viscosity, its reciprocal, relative fluidity, was calculated and plotted against protein concentration, in g. per liter as abscissas, since the fluidity of many proteins,[19] including SA, is a linear function of concentration. The fluidities listed in Table I are those at 1% protein concentration. Nitrogen and phosphorus analyses were made after each series of viscosity measurements, and the protein concentration was calculated, allowing for the phosphoryl content.

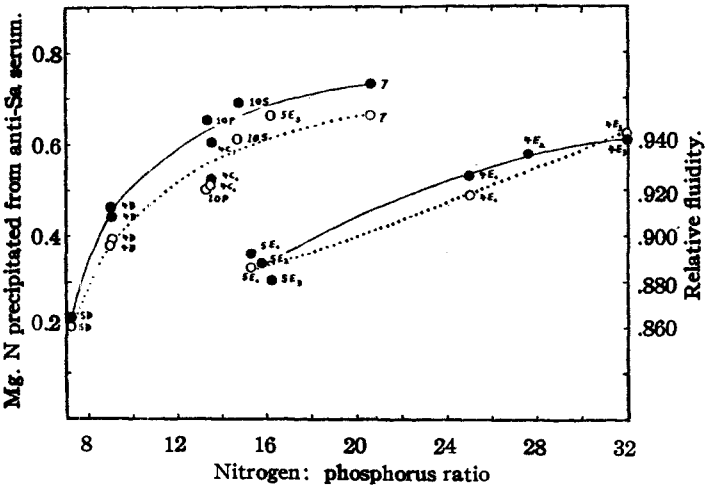


Fig. 4.—Fluidity of PSA and precipitin cross reaction with anti-SA as functions of the N:P ratios of PSA preparations: Fluidity, open circles; Precipitin reaction, solid circles. The PSA preparation is indicated by the number next to each point. PSA preparations 4B, 4D, 5B, 7, 10S and 10P were studied at a time when they still contained all the phosphorus introduced in their preparation. A solid line has been drawn through the precipitin reaction data and a dotted line through the fluidities (in order to show the correlation between these properties). PSA preparations $4E_1$, $4E_2$, $4E_3$, $5E_1$, $5E_2$ and $5E_3$ which were partially dephosphorylated, fall into a separate group (lower right of graph) and separate solid and dotted lines have been drawn for the data on these preparations. PSA$4C_1$, C_2, and $5E_2$ show exceptional behavior (see text).

The data given in Fig. 3 show that the fluidity of SA is decreased by phosphorylation, *i. e.*, the viscosity is increased. This may be due either to greater molecular asymmetry or to greater hydration of PSA. Egg albumin behaved similarly on phosphorylation.[2] Denaturation of proteins by various agents also results in decreased fluidity.[20] A close connection between the content of phosphoryl groups and fluidity is shown by the data given in Fig. 4, in which the fluidities at 1% protein concentration of a number of PSA preparations are plotted against their N:P ratios. Fluidity decreases with increasing phosphorus content, and the curve drawn fits most of the data fairly well. It is apparent that, with the exception of PSA$5E_3$, products obtained by dephosphorylation at 37° possess lower fluidities than would be expected from the loss in phosphorus. The role of secondary changes which fail to reverse on dephosphorylation will be discussed in connection with parallel changes in immunological specificity. The soluble fraction of the dephosphorylated PSA5E, which showed exceptional behavior, was possibly the least denatured of the various products considered in this connection.

The viscosity of a protein solution is believed to reflect the particle shape and the extent of hydration of the protein. Since it is not possible, at present, to evaluate the contribution of the hydration factor, the molecular shape cannot be determined uniquely. It was shown by Treffers[19] that the fluidity of phosphorylated egg albumin varied slightly with *p*H. In the case of PSA the fluidity was also found to depend slightly on *p*H (*cf.* lines 4 and 5, Fig. 3). When the fluidity of PSA14 was determined in two borate buffers ($\mu = 0.01$) at *p*H 8, one containing 0.05 *M* and the other 0.20 *M* sodium chloride, the fluidities at 1% protein concentration were found to be 0.902 and 0.922, respectively. Whether this small but significant difference is due to suppression of an electroviscous effect at the higher salt concentration or to a reduction in hydration cannot be stated.

10. Sedimentation Constant.—Determination of the sedimentation constant of PSA14 at 0.8% concentration in *p*H 8 borate buffer yielded the value $S^{20}_{H_2O}$ 4.5, the same as that obtained for SA.

11. Electrophoretic Data.—The mobilities of PSA10A and PSA10B in *p*H 7.9 borate–sodium chloride buffer of ionic strength 0.16 (8.50 g. sodium chloride, 2.97 g. boric acid, 1.14 g. sodium borate per liter of solution) were -9.1 and -9.7×10^{-5} cm.²/sec. volt, respectively (protein concentration 0.36 and 0.31%, respectively, field strength 4.0 volt/cm.), with a small, slightly faster component ($U = -9.9$ and -10.3, respectively) appearing in both fractions on the ascending side only.

The mobility of PSA14 in borate–sodium chloride buffer of *p*H 8.0 and $\mu = 0.16$ (0.4% protein concentration) was -8.4. At *p*H 3.5 and 4.3 the mobilities were $+3.3$ and -2.2, respectively. These values indicated an isoelectric point near *p*H 3.9, in agreement with data on comparable preparations by the method of minimum solubility.

Immunological Properties of PSA and their Relation to Chemical and Physical Characteristics

Two rabbits weighing about 3 kg. each were immunized by intravenous injections, four times weekly, of alum-precipitated crystalline horse serum albumin. Each animal received a total of 122 mg. of protein in courses of 24 and 16 injections with two weeks intervening. Bleedings were taken on the sixth or seventh day after the last injection. Three other rabbits were injected intravenously with alum-precipitated PSA2 and PSA4, each receiving 162 mg. of protein in courses of 16, 16 and 15 injections.

With PSA2′ and PSA4B as antigens, the two strongest anti-PSA rabbit sera, 733_1 and 733_2, were analyzed for precipitin nitrogen by the quantitative method.[21] Increasing quantities of antigen were added to 1- or 2-ml. portions of serum and the amount of nitrogen precipitated from each was plotted against the antigen added. In this way, a precipitin curve of the PSA–anti-PSA reaction was

(19) H. P. Treffers, THIS JOURNAL, 62, 1405 (1940).

(20) Reference 18, p. 416.

(21) M. Heidelberger and F. E. Kendall, *J. Exp. Med.*, 62, 697 (1935). Details of the analytical methods used will be found in this article.

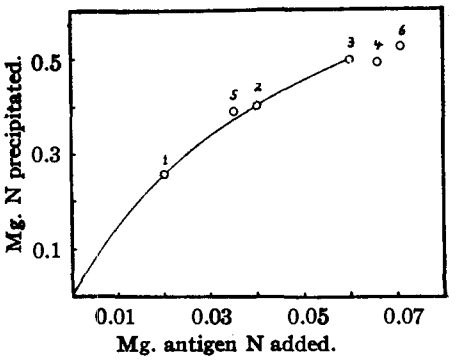


Fig. 5.—Precipitin reaction of SA and PSA with 1.0 ml. of rabbit anti-PSA serum 733_8. Points 1, 2 and 3 represent PSA2′; point 4, PSA4B; points 5 and 6, SA. (See Table IV.)

obtained (Fig. 5). This resembled that of SA–anti-SA closely,[22] except that supernatant fluids in the "equivalence zone" gave positive reactions for both antigen and antibody.

TABLE IV

PRECIPITIN ANALYSES OF ANTI-PSA SERUM 733_8

Analysis no.	Antigen	Antigen N added per ml. of serum, mg.	N pptd. from 1 ml. of serum, mg.	Reaction of supernatant tested with SA	PSA	anti-SA	anti-PSA
1	PSA2′	0.020	0.26		+++		−
2	PSA2′	.040	.40		+++		−
3	PSA2′	.060	.49		+≐		+
4	PSA4B	.066	.49	++	−	++	++
5	SA	.035	.39	−	++	−	−
6	SA	.071	.52	−	−	++	++

Culbertson[23] and Kendall[24] have stated that positive tests for both antigen and antibody are not given by the same supernatant fluid in a system containing a single antigen and its homologous antibody. Positive tests for both furnish evidence that the antiserum contains more than one kind of antibody and that the antigen is a mixture of antigenic components.[23,24] PSA, therefore, does not behave like a single antigen.

Anti-PSA serum 733_8 was also tested with SA at a point in the antibody excess zone and at one in the equivalence zone, with the result that the cross-reacting antigen (SA) precipitated as much nitrogen as the homologous antigen (PSA2). This antiserum to PSA, therefore, did not distinguish between SA and PSA (Fig. 5).

The reciprocal cross reaction, that is, between PSA and anti-SA, was studied with a serum pool of about 200 ml. which had previously been absorbed with 28 mg. of twice-precipitated horse euglobulin[25] in order to remove anti-globulin.[26] For the test of each preparation 1.0-ml. portions of serum were usually allowed to stand with about 0.05 and 0.10 mg. of PSA nitrogen at 0° for four days. Under these conditions PSA may be considered to remain

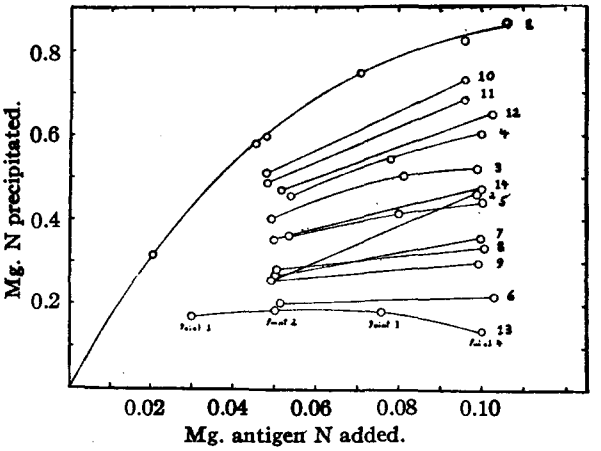


Fig. 6.—Precipitin reaction of SA and PSA with 1.0 ml. of rabbit anti-SA serum: curve 1, SA; curve 2, PSA4B; curve 3, PSA4C$_1$; curve 4, PSA4C$_2$; curve 5, PSA4D; curve 6, PSA5B; curve 7, PSA5E$_1$; curve 8, PSA5E$_2$; curve 9, PSA5E$_3$; curve 10, PSA7; curve 11, PSA10S; curve 12, PSA10P; curve 13, PSA13 (See text for explanation); curve 14, PSA13A.

unaltered for the duration of the precipitin test. The results, plotted in Fig. 6, indicate marked differences of specificity between SA and PSA, as well as between different lots of PSA. Depending upon the extent of phosphorylation, different preparations of PSA precipitated from 20 to 90% of the total antibody to SA. Since only a fraction of the anti-SA was precipitated by the various lots of PSA, no matter how much antigen was used, the absence of admixed SA in PSA seemed certain. The PSA–anti-SA reaction may therefore be used as a means of studying specificity changes due to phosphorylation.

Kabat and Heidelberger[22] found that R-salt-azobiphenyl-azo-SA (dye-SA) reacted like SA with anti-SA but that SA reacted only partially with anti-dye-SA. The groupings to which the dye had coupled were apparently not concerned with the specificity of SA, since their modification was not detectable in anti-SA serum. Phosphorylation, on the other hand, seems to affect important groupings, since it causes marked changes in specificity. Inability to distinguish between PSA and SA by means of anti-PSA would seem to contradict this interpretation, were it not for the observation that PSA may be largely dephosphorylated merely by exposure to 37°. Since phosphate is easily lost *in vitro* in the absence of enzymes, it might be split off even more rapidly and extensively in the animal organism under the action of phosphatases. It is therefore possible that on immunization of rabbits with PSA, both spontaneous and enzymatic dephosphorylation converted some of the PSA into SA, leading to the production of anti-SA as well as anti-PSA by the rabbit. If this interpretation be accepted, the instability of PSA would render a direct study of its specificity difficult.

From Fig. 6, in which the cross reactions of all preparations of PSA with anti-SA are plotted, and from Fig. 4, in which the cross reactions are plotted in relation to the phosphorus content of the preparations, it is evident that the specificity of SA has been altered by phosphorylation to an extent which depends on the amount of phosphorus introduced. This is brought out by the positions of the curves in Fig. 6 and by the fairly smooth curves which may be drawn through the points in Fig. 4. This conclusion applies mainly to primary phosphorylation products, since partial dephosphorylation (prepns. 4E$_1$, 4E$_2$, 4E$_3$, 5E$_1$, 5E$_2$, 5E$_3$) does not result in as great a change toward the SA curve as would be expected from the loss of phosphorus.

Although similar quantities of antigens were used as in tests with the other preparations, three of the four points plotted for PSA13, the preparation of highest phosphoryl

(22) E. A. Kabat and M. Heidelberger, *J. Exp. Med.*, **66**, 229 (1937).

(23) J. T. Culbertson, *J. Immunol.*, **28**, 279 (1935).

(24) F. E. Kendall, *J. Clin. Invest.*, **16**, 921 (1937).

(25) A Felton solution, prepared as follows, was used: Horse anti-pneumococcus types I and II serum was poured into 20 volumes of chilled 0.001 *M* phosphate buffer of *p*H 5.6. The resulting euglobulin precipitate was dissolved in a small volume of 0.9% sodium chloride solution and was reprecipitated as described above.

(26) N. E. Goldsworthy and G. V. Rudd, *J. Path. Bact.*, **40**, 169 (1935).

content (Fig. 6, lowest curve), lie in the antigen-excess zone. The supernatants, when tested with antiserum, gave tests of + for point 2 and ++ for points 3 and 4. The rate of reaction of PSA13 with anti-SA was so slow that the point 2 analysis required six days for completion of precipitation, while that at point 4 took eight days. The last part of the curve shows partial inhibition of precipitation, although the amount of antigen used would lie in the equivalence zone of the homologous reaction (Fig. 6, top curve). Further increase of antigen to 0.34 mg. of nitrogen resulted in complete inhibition. Although inhibited with respect to PSA, the supernatant from the point 4 analysis reacted strongly and immediately with SA. This shows that PSA13 combined with only a small fraction of the total antibody, leaving the rest free to react with SA.

The correlation between N:P ratio and extent of cross-reaction, as shown in Fig. 4, parallels that between N:P ratio and fluidity. The basis for this relationship may lie in changes of molecular surface structure as a result of alterations in molecular shape, for the fluidity of a protein is a function either of hydration or shape, while immunological specificity is an expression of molecular surface configuration.

In one qualitative test PSA2 gave a weak precipitin reaction with an antiserum to phosphorylated egg albumin,[2] PEa, but the reciprocal test, that is, between PEa and anti-PSA, was negative.

Discussion

Perhaps the most marked chemical difference between the synthetic phosphoprotein PSA and the natural phosphoproteins lies in the instability of the phosphoryl groups. Caseinogen does not lose phosphorus spontaneously but is dephosphorylated on treatment with 1% sodium hydroxide for twenty-four hours at 37°.[8] In sharp contrast, PSA loses much of its phosphorus spontaneously even in the cold but, on the other hand, some of the phosphoryl groups resist splitting by 1% alkali at 37°.

The phosphorus content of PSA considerably exceeds that of casein,[27] but the 2.7% of phosphorus contained in the most heavily phosphorylated preparation, PSA13, still falls far short of the maximum number of phosphoryl groups with which SA could combine. Since a molecule of SA contains 1 tryptophan, 18 tyrosine, 32 serine, 35 threonine, 22 arginine, 20 histidine residues and 75 free amino groups, there is a total of at least 200 groups[28] potentially reactive with phosphorus oxychloride, but less than one-third (61 groups) of these were phosphorylated in PSA13 under the preparative conditions used. About 27 phosphoryl groups were apparently linked to amino groups as shown by analyses according to Van Slyke. Other lots, also prepared in the presence of strong alkali, contained 34–46 atoms of phosphorus, while preparations made with the aid of borate buffer never exceeded a phosphorus content of 32 atoms, even when a more alkaline (*p*H 10.3) borate–carbonate buffer was used (PSA11). The more complete phosphorylation with sodium hydroxide is probably due to the high alkalinity produced locally upon addition of each drop of strong alkali. The amount of phosphorus oxychloride usually employed, 225 moles per mole of SA, was apparently sufficient for maximal reaction, since not much more phosphorus could be introduced by doubling or quadrupling it. The variable phosphorus content of different sodium hydroxide preparations made under apparently identical conditions is believed partly due to spontaneous phosphorus loss during purification and partly to *p*H fluctuations, possibly local, unavoidable in a discontinuous neutralization technique. The phosphorus content of lots made with borate buffer was actually more constant since lots 6 and 7, both made in the presence of sodium tetraborate, had N:P ratios of 21.6 and 20.6, respectively, while lots 8 and 10, prepared with the aid of the more soluble potassium tetraborate, had ratios of 14.6 and 14.7. Lot 14, prepared with potassium tetraborate, contained somewhat more phosphorus.

Changes in immunological specificity which occur on chemical substitution of a protein depend not only on the nature of the radical introduced and its site and mode of attachment, but also on the protein itself. Kabat and Heidelberger[22] found that dye-SA was quantitatively identical with SA in its reaction with rabbit antiserum to SA, while egg albumin (Ea) coupled to the same dye scarcely reacted with anti-Ea serum. Similarly, PSA reacted much more strongly with anti-SA than PEa did with anti-Ea although both proteins had been given the same treatment and the products had been isolated by identical methods. Analogous differences between SA and Ea appear on denaturation (Dn). While the specificity of DnSA is not much different from that of SA,[29] DnEa has a specificity radically different from that of Ea.[30] It does not seem far-fetched to seek a relation between the different behavior of the two proteins on denaturation and their unlike response to chemical substitution.

To study the influence of chemical alteration on the complex immunological specificity of proteins it is necessary to consider the effect on the surface structure of the molecule. Since it is generally agreed that immunological specificity depends on the configuration of the molecular surface,[31] structural surface changes should be reflected in altered specificity. In general, two kinds of surface changes may result from any chemical alteration of so large a structure as a protein molecule. Wherever a reactive group like hydroxyl, amino or guanidino combines with a foreign radical, a primary local change ensues, due to the elimination of the reactive protein group from the surface and its replacement by the foreign radical as a new constituent of the surface. Thus the protein molecule acquires a new con-

(27) Reference 16, p. 341.

(28) From E. Brand, B. Kassel and L. Saidel, *J. Clin. Invest.*, **23**, 437 (1944). Our value for total NH_3 N, 1.2%, is lower than Brand's possibly due to the shorter reaction time employed.

(29) J. O. Erickson and H. Neurath, *J. Exp. Med.*, **78**, 1 (1943).

(30) C. F. C. MacPherson and M. Heidelberger, THIS JOURNAL, **67**, 585 (1945).

(31) For a recent summary, see L. Pauling, D. H. Campbell and D. Pressman, *Physiol. Rev.*, **23**, 203 (1943).

figuration at these specific sites and as a result may change its serological specificity, the extent of the change depending upon the number and kind of groups introduced. In addition, the chemical substitution may cause general changes in the architecture of the protein molecule as a result of the disruption of certain structurally important links, possibly hydrogen bonds, involving hydroxyl and amino groups. In this way a radically different molecular surface topography might result, with consequent marked change of serological specificity. In the case of SA there are only limited specificity changes due to the primary local reaction, while with Ea an upheaval of topography due to secondary changes may be postulated.

The preparation of PSA by two different methods, one employing strong alkali, the other potassium or sodium borate, affords an opportunity to detect specificity changes due to exposure to strong alkali. Inspection of Fig. 4, however, shows that products of the same N:P ratio have similar viscosities and specificities whether sodium hydroxide or borate was used. The superimposition of alkali denaturation in PSA seems unlikely when the phosphorylated protein is prepared in the cold as in the present study.

The ease with which PSA may be partially dephosphorylated furnishes an approach to the question of general structural changes. If the phosphorylation had caused only primary local changes which should be reversible on dephosphorylation, the physical and immunological properties of partially dephosphorylated PSA should return toward those of SA in proportion to the loss in phosphorus. An increase of cross-reactivity in anti-SA is indeed observed after partial dephosphorylation, but its magnitude is not commensurate with the loss in phosphorus. The cross reaction of different products dephosphorylated at 37° plotted against the N:P ratio (Fig. 4) falls below that of products of equal initial phosphorus content. PSA4C_1 and PSA4C_2, which were dephosphorylated at 0°, do not show this effect as clearly, since the loss of phosphorus at 0° was relatively small. Fluidities plotted against N:P ratios (Fig. 4) show the same thing, indicating also that general structural alterations take place and that these are irreversible on dephosphorylation.

The failure of the phosphoryl group to confer a $-PO_3H_2$ specificity upon SA, as indicated by lack of cross reactions between PSA and PEa and their antisera need not mean that the phosphoryl group is incapable of acting as a hapten or determinant of specificity. A possible explanation may be sought in the instability of PSA discussed in an earlier paragraph.

The parallel between physical and immunological properties, apparent from the data plotted in Fig. 4, suggests that both are functions of the same chemical factors. While the fluidity or viscosity of PSA depends somewhat on *p*H and salt concentration, essentially it is a function of molecular shape. A change of shape is reflected by a viscosity change, if constant hydration be assumed. Since a change in shape is necessarily accompanied be an alteration of molecular topography, there might also occur a change in specificity. Thus, a physical transformation, following upon a chemical change, might find expression in a corresponding change in immunological specificity. The close correlation between fluidity and specificity shown in Fig. 4 suggests that such might be the case with PSA.

Summary

Crystallized horse serum albumin was phosphorylated with phosphorus oxychloride in the cold. The physical, chemical and immunological properties of the substitution products obtained under different conditions were studied and compared with those of serum albumin. Due to the introduction of the acidic phosphoryl groups, the isoelectric point was lowered and many preparations became insoluble in its neighborhood. The buffering capacity was increased for both acid and alkali and, at suitable *p*H values, the protein migrated more rapidly in an electric field. The serological reactivity with anti-SA serum and the relative fluidity were reduced in proportion to the phosphorus content.

Chemical studies indicated that some of the phosphoryl groups were coupled to amino groups of the protein while others were probably esterified with tyrosine residues.

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